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14. ABSTRACT

The purpose of phase (II) of the study was to investigate the effects of local wound management, specifically pulsatile lavage and closure through the zone of blast injury versus debridement above the zone of injury, on the prevalence and severity of ectopic bone formation following extremity blast amputation in a rat model. Thirty-six Sprague-Dawley rats underwent hindlimb blast amputation via a column of propelled water following detonation of a submerged explosive. There were three treatment groups (each n=12): Group 1 was irrigated with 1L of diluted chlorhexidine solution using pulsatile lavage and closed through the zone of blast injury; Group 2 underwent syringe irrigation and debridement above the zone of injury (ZOI) with through-knee amputation; and Group 3 underwent both pulsatile lavage and through-knee amputation. The control group (n=12) was irrigated with 250ml of chlorhexidine solution using a bulb syringe and minimal debridement of skin edges. The animals were followed with serial AP and lateral radiographs until euthanasia at 24 weeks, at which time HO severity was quantified as (0) absent, (1) mild, (2) moderate, or (3) severe, and HO type was qualified as contiguous with the residual limb or as distinct ectopic bony islands. No HO contiguous with the amputated residual limb occurred in any animal that underwent through-knee amputation above the zone of injury compared with a high prevalence of HO in the residual limbs closed through the zone of injury, suggesting that the local inflammatory milieu plays a critical and essential role in HO induction. Animals treated with pulsatile lavage developed more HO than animals amputated at a comparable level and irrigated with a bulb syringe; high-pressure irrigation appeared to potentiate the HO response and deserves further study.

15. SUBJECT TERMS

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INTRODUCTION:

In the recent conflicts in Iraq and Afghanistan, >80% of military personnel sustained an extremity injury, of which approximately 66% were attributed to blast (IED) mechanism. Enhancement in body armor technology and medical treatment has led to increased survival following extremity injury/amputation. However, having survived the initial trauma and resulting limb amputation, soldiers face complications such as development of heterotopic ossification (HO) within the residual limb causing pain, overlying skin and muscle breakdown, poor prosthetic fitting and function, a need for surgical revision of the residual limb, and delayed rehabilitation. Heterotopic ossification is the pathologic formation of mature, lamellar bone within non-osseous tissues. This abnormal growth results from a disturbance in the regulation of normal skeletogenesis. Between 2001-2005, greater than 60% of combat amputees developed HO. Current treatment options are limited and are restricted to surgical excision of the offending bone rather than primary prevention. Investigation of the effects of blast trauma on the musculoskeletal system, specifically the prevention of HO in the residual limbs of blast amputees, is a priority in the care of our war-wounded veterans. To this end, after refinement of a reliable and reproducible model of HO after blast amputation (phase I), the focus of this study was to investigate the effects of local wound management (phase II), specifically pulsatile lavage and debridement above the zone of injury, and various prophylactic measures (phase III) on the formation of ectopic bone following extremity blast amputation in Sprague-Dawley rats using a previously established animal model.

BODY:

Phase I: The work conducted and completed in the first year (phase I) of this study addressed specific aims 1 and 2.

Specific aim 1: We will investigate the effect of *blast medium* on the development of ectopic bone in the amputation stump, specifically comparing a water blast versus a soil-blast in the SD rat. We anticipate greater soft tissue damage and hence predict greater ectopic bone formation with the soil blast.

Specific aim 2: We will investigate the effect of *anatomical location* on the development of ectopic bone in the amputation stump, specifically comparing forelimb and hindlimb amputations in the SD rat. We anticipate greater ectopic bone formation in the hindlimb injury, related to poorly understood humoral mechanisms.

In phase I, *all* animals developed some degree of heterotopic ossification following blast amputation; the severity and pattern was more dependent upon limb selection than blast medium.

After euthanasia at twenty-four weeks, three independent observers reviewed the series of radiographs and graded the severity of heterotopic bone as either absent, mild, moderate, or severe based on a modification of the scale used by Potter et al. for human amputees. Heterotopic ossification was considered to be mild if it measured less than 25% of the width of the tibial plateau on either the anteroposterior or lateral view radiograph, moderate if it measured 25% to 50% of the width of the tibial plateau on either radiograph, or severe if it measured more than 50% of the width of the tibial plateau on either radiograph. Each severity grade was assigned a corresponding numerical value (absent = 0, mild = 1, moderate = 2, severe = 3). The ectopic bone was also classified as one of three *types*. Type 1 was defined as bone contiguous with the stump while remaining within the normal bony configuration. Type 2 was defined as ectopic bone contiguous with the bony stump but outside the normal bony envelope. Type 3 was defined as ectopic bone originating within the surrounding soft tissues of the residual limb, but not contiguous with the bony stump. In the grading scales for both severity and type of HO, the score assigned with greatest frequency (i.e., by two of three raters) was used in the statistical analysis. Cohen's kappa statistic was used to determine inter-rater reliability of observations (both severity and type) based on radiographs among the three independent raters.

Statement of Work Task 1: Approval process for animal studies (2 to 4 months), followed by Regulatory and Programmatic reviews (2 to 4 months).

- Regulatory approvals were obtained and have been maintained through our local IACUC as well as ACURO throughout the period of this work.

Statement of Work Task 2: Achieve consensus on whether sand or water blast medium results in a higher prevalence of HO. Perform previously established blast amputation protocol ($n=48$ rats). 24 rats to undergo blasting protocol with a water medium and 24 rats to undergo blasting with sand medium. Observe rats with radiographs (at 10 days and 4, 8 12, 16, 20 and 24 weeks) for changes associated with classification system described by Potter et al.

- There was no difference in observed HO between the water and sand groups. Both the water (mean 2.083) and sand (mean 2.083) hind limbs developed the same severity of HO; this was also seen with the forelimbs (mean water 1.0833, mean sand 1.333) (Figures 1,2). However, the sand group developed more wound complications than the water group resulting in reoperations for revision of the wound. Future studies will use water as a blast medium in order to control for external variables that could influence the development of HO.

Statement of Work Task 3: Achieve consensus on whether forelimb or hind limb amputations result in higher prevalence of HO. Perform previously established blast amputation protocol ($n=48$ rats). 24 rats to undergo forelimb amputation and 24 to undergo hind limb amputation. Observe rats with radiographs (at 10 days and 4, 8 12, 16, 20 and 24 weeks) for changes associated with classification system described by Potter et al.

- Compared with the forelimb, hind limb blast amputation stumps developed more severe heterotopic bone and were more likely to exhibit ectopic bony islands in the surrounding soft tissues in this animal model. Hind limb stumps developed one full grade more severe heterotopic ossification (mean 2.0833) than did the forelimb amputations (mean 1.0833) (Fig 1). When comparing heterotopic ossification type based on plain radiographs, ten hind limbs developed type 3 ectopic bone compared with only four forelimbs (Fig 2). These observed differences correlate closely with clinical observations in humans. Forsberg et al. reported an incidence of ectopic bone formation in lower and upper extremity wartime amputations of 66% and 30%, respectively. While studying a different mechanism of amputation, Helm and Walker reported that 78% of upper extremity electrical burn amputations developed substantial ectopic bone compared with 90% of lower extremity amputations.

Phase II: The work conducted and completed in the second year (phase II) of this study addressed specific aim 3.

Specific Aim 3: We will investigate the effect of *local wound care*, specifically;

- a) ...the influence of *pulsatile lavage* on the prevalence and severity of heterotopic bone formation in the blast-injured amputated extremity. We hypothesize that pulsatile lavage will *augment* the process of heterotopic ossification as a result of increased soft tissue microdamage.
- b) ...the influence of *aggressive local soft tissue debridement* on the prevalence and severity of heterotopic bone formation in the blast-injured amputated extremity. We hypothesize that aggressive debridement (above the zone of injury) will *diminish* the process of heterotopic ossification by reduction of the burden of damaged soft tissue.

In the second year (phase II) of this study, the development of HO varied substantially according to the treatment group to which the animals were assigned. The work conducted and completed in year two (phase II) of the grant addressed;

Statement of Work, Task 4: (Phase II) Investigate the influence of pulsatile lavage on the prevalence and severity of HO. Perform hind limb blast protocol with water, adjusted from SA 1&2 (n=12 rats). All 12 will undergo irrigation with pulsatile lavage and closure through the zone of injury. Observe rats with radiographs (at 10 days and 4, 8, 12, 16, 20 and 24 weeks) for HO changes associated with classification system described by Potter et al. Compare data to rats treated with bulb syringe irrigation in SA 1&2.

Statement of Work, Task 5: (Phase II) Investigate the influence of aggressive local soft tissue debridement on the prevalence and severity of HO. Perform hind limb blast protocol with water, adjusted from SA 1&2 (n=24 rats). All 24 animals will undergo aggressive debridement in the form of above knee amputation above the zone of injury; 12 will have the amputation site irrigated by bulb syringe and 12 will have irrigation by pulsatile lavage. Observe rats with radiographs (at 10 days and 4, 8, 12, 16, 20 and 24 weeks) for changes associated with classification system described by Potter et al. Compare data between rats with different methods of irrigation after through-knee amputation, as well as to rats treated with closure in zone of injury in SA 1&2.

- The mean HO severity in the control group (hind limb blast, syringe irrigation, zone of injury closure) at 24 weeks was 1.75, compared to 1.58 in pulsatile lavage group ($p=0.6278$), 0.33 in the through-knee amputation group with syringe irrigation ($p<0.0001$), and 1.83 in the through-knee amputation plus pulsatile lavage group ($p=0.8126$) (Figure 3). Five of 12 (42%) control group animals developed HO contiguous with the stump plus ectopic bony islands compared to eleven of 12 (92%; $p=0.0272$) in the pulsatile lavage group with closure in the zone of injury (Table 1). Among animals treated by through-knee amputation above the zone of injury, 4 of 12 (33%) animals receiving bulb syringe irrigation developed isolated ectopic bony islands compared to 11 of 12 (92%) in the group managed with pulsatile lavage ($p=0.0094$) (Table 2). Three animals in pulsatile lavage groups had wound dehiscence in the immediate postoperative period; two had wound breakdown without signs of infection several weeks after the blast procedure and required simple re-closure without additional debridement.
- Pulsatile lavage groups exhibited two types of radiopaque lesions in the soft tissues: early lesions were characterized by fluffy radiopaque densities with histologic evidence of dystrophic calcification and tissue necrosis, while late lesions exhibited dystrophic calcification with chondrocytes characteristic of HO. Pulsatile lavage increased the severity and type of HO.

Phase III: With the start of year 3, we have begun phase III experiments, which address specific aim 4.

Specific Aim 4: We will investigate the effect of *therapeutic interventions to mitigate the formation of heterotopic bone*, specifically;

- a) ...the influence of *nonsteroidal anti-inflammatory medication* on the prevalence and severity of heterotopic bone formation in the blast-injured amputated extremity. We hypothesize that systemic administration of Naproxen sodium, with a 24 hour delay in institution of therapy after injury and a 7 day treatment period, will *diminish* the process of heterotopic ossification in the injured limb stump.
- b) ...the influence of *external beam radiation* on the prevalence and severity of heterotopic bone formation in the blast-injured amputated extremity. We hypothesize that radiation therapy, 800cGy delivered within 72 hours of injury, will substantially *diminish* heterotopic ossification in the injured limb stump.

Statement of Work, Task 6: (Phase III) Investigate the influence of NSAID therapy on the prevalence and severity of HO. Perform hind limb blast protocol with water adjusted from SA 1&2 (n=12 rats). All 12 will undergo treatment with Naproxen sodium 24 hours

after injury for 7 days. Observe rats with radiographs (at 10 days and 4, 8 12, 16, 20 and 24 weeks) for changes associated with classification system described by Potter et al. Compare data to control rats with no NSAID treatment in SA 1&2.

Statement of Work, Task 7: (Phase III) Investigate the influence of external beam radiation on the prevalence and severity of HO. Perform hind limb blast protocol with water, adjusted from SA 1&2 (n=12 rats). All 12 will undergo exposure to 800cGy within 72 hours of injury. Observe rats with radiographs (at 10 days and 4, 8 12, 16, 20 and 24 weeks) for changes associated with classification system described by Potter et al. Compare data to control rats treated without radiation in SA 1&2. (1 month)

- Blasting for Phase III animals has begun with the commencement of year 3, and the animals are receiving either Naprosyn or radiation prophylaxis at this time per protocol without substantial complications.

KEY RESEARCH ACCOMPLISHMENTS:

- Work concerning specific aims 1-3 and statement of work tasks 1-5 has been completed in a timely fashion and according to the proposed schedule.
- Hind limb blast with water medium provided the animal model that most reproducibly resulted in HO after blast amputation without complicating infection of the local wound.
- Amputation *above the zone of injury* substantially mitigated, if not eliminated, the HO response to blast amputation.
- Pulsatile lavage was associated with radiodensity within the soft tissues of the irrigated limb;
 - Early reaction to pulsatile lavage histologically represented dystrophic calcification with substantial spontaneous resolution over time.
 - Persistent late reaction to pulsatile lavage histologically contained HO with characteristic chondrocytes

REPORTABLE OUTCOMES:

- Poster and podium presentations at Eastern Orthopaedic Assn (10/2011), AAOS (2/2012), Orthopaedic Research Society (2/2012), and Eastern Orthopaedic Assn (6/2012).
- Abstracts submitted for annual meetings to AAOS (2013) and ORS (2013).
- Manuscript from Phase I work submitted to JBJS and under review/revision (appendix).
- Two full CDMRP PRORP TRPA applications submitted for September 25, 2012 deadline:

OR120070; Optimizing Function and Preventing Heterotopic Ossification after Blast Injury: Role of Amputation Level, Bone Management, and Pulsed Lavage in an Animal Model.

OR 120071; Early Identification of Molecular Predictors of Heterotopic Ossification following Extremity Blast Injury: Animal Model Correlation with Human Disease.

CONCLUSIONS:

Two principal observations from our Phase II work have important implications for immediate translation to clinical practice and may represent substantial advancements in clinical care.

- *Heterotopic ossification following blast amputation is substantially mitigated, if not eliminated, when amputation is performed above the zone of injury.* This observation may represent an important initial wound management strategy that is most applicable to mid to distal tibia blast amputations, where primary below knee amputation can be performed above the zone of injury while still retaining a functional knee joint. A full DoD PRORP TRPA application has been submitted on this subject for the July 29, 2012 deadline.
- *The use of pulsatile lavage is associated with incremental soft tissue injury characterized by dystrophic calcification in the blast injured limb and, in some cases, potentiates the HO response in the residual limb.* This observation may have immediate implications for clinical practice and supports abandonment of pulsatile lavage in blast wounds. While more pronounced HO was observed on plain XR and CT with pulsatile lavage, a modified Potter scale was not sufficiently sensitive to discern any difference between the groups. Future studies will use CT to quantitatively assess ectopic bone and more accurately determine the effects of pulsatile lavage on HO.

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APPENDIX

Supporting Data:

Figure 1: HO Severity – According to Limb and Blast Medium

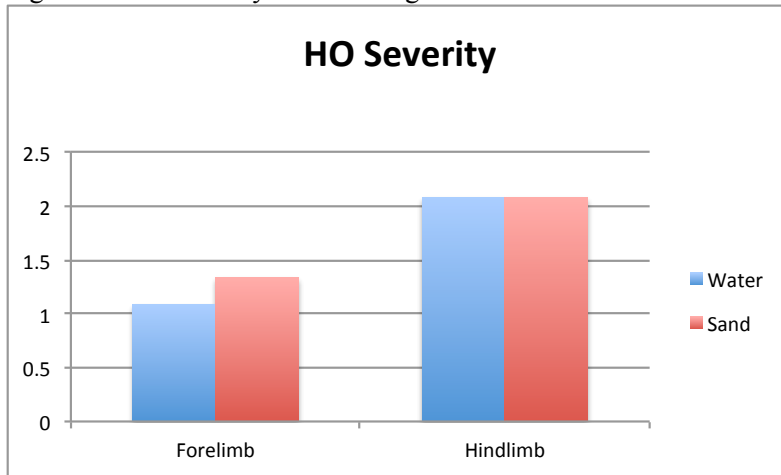


Figure 2: HO Type – According to Limb and Blast Medium

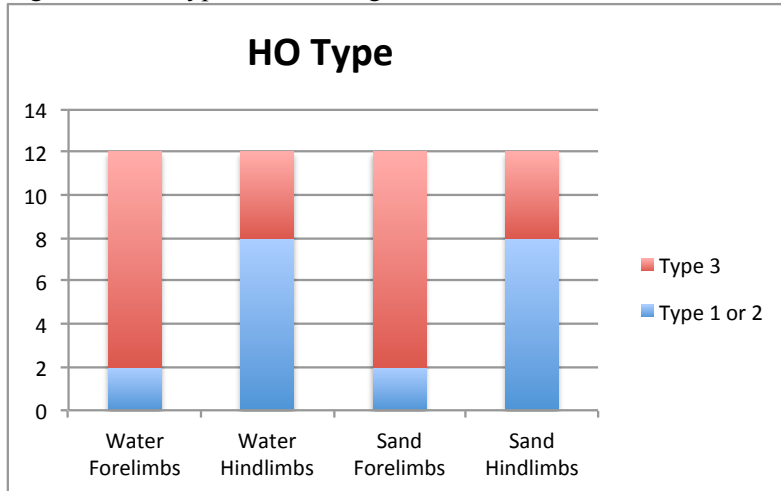


Figure 3: HO Severity – According to Local Wound Care
 Severity scores scale: absent (0), mild (1), moderate (2), and severe (3).
 Error bars represent standard deviation.

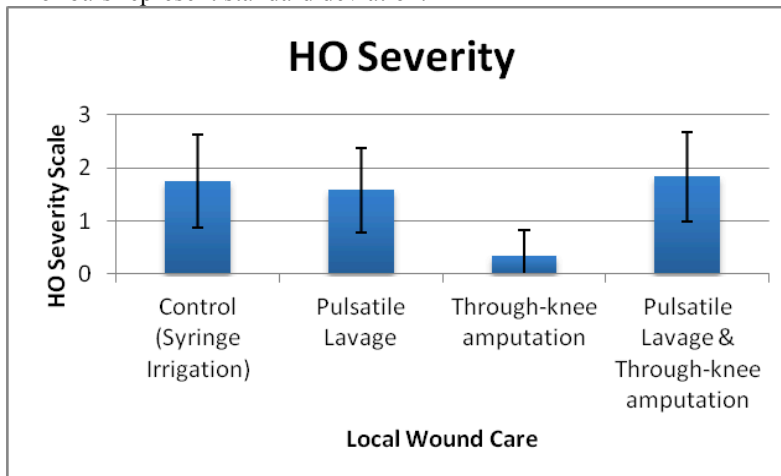


Table 1: HO after Zone of Injury Closure: Syringe Irrigation vs. Pulsatile Lavage
Overall Inter-observer Reliability: $k=0.79$

	No. (out of 12) with Type 3 HO (ectopic bony islands)
Control (Syringe Irrigation)	5
Pulsatile Lavage	11 ($p= 0.0272$)

Table 2: HO after Through-knee Amputation: Syringe Irrigation vs. Pulsatile Lavage
Overall Inter-observer Reliability: $k=0.88$

	No. (out of 12) with Type 3 HO (ectopic bony islands)
Syringe Irrigation & Through-knee Amputation	4
Pulsatile Lavage & Through-knee Amputation	11 ($p=0.0094$)

Journal of Bone and Joint Surgery

Influence of Limb Selection and Blast Medium on Heterotopic Ossification after Blast Amputation in a Rat Model --Manuscript Draft--

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Abstract:	<p>Background: Heterotopic ossification develops after nearly two-thirds of traumatic blast amputations that occur in the contemporary battlefield. We used a Sprague-Dawley rat model, without the need for addition of an exogenous osteogenic agent, to evaluate the pathophysiology of ectopic bone formation in a traumatic blast amputation setting. We investigated the comparative frequency, quality, and quantity of heterotopic bone developing after blast amputation under various conditions.</p> <p>Methods: Forty-eight animals underwent blast amputation and primary surgical closure (twenty-four forelimbs and twenty-four hind limbs), with only marginal skin débridement. Half of the amputations in each designated limb group were subjected to blasted sand, and the other half were blasted with water. All animals were followed with serial radiographs until euthanasia at twenty-four weeks. Heterotopic bone severity and type were assessed at eight, sixteen, and twenty-four weeks by three independent graders using a novel grading scale.</p> <p>Results: All animals developed radiographic evidence of heterotopic ossification without addition of exogenous osteogenic material. Overall, hind limbs developed more severe and more extensive ectopic ossification than did forelimbs, as evidenced by a greater propensity to develop ectopic bony islands in the soft tissues. No differences were observed in ectopic bone development when comparing blast amputation with sand versus blast amputation with water.</p> <p>Conclusions: Hind limb amputation sites were more likely to develop severe</p>

	heterotopic ossification and ectopic bony islands within the adjacent soft tissues than were forelimb amputations. Despite a trend toward increased risk of infection, sand-blasted animals did not develop substantially more ectopic bone than water-blasted animals. The grading scale developed in this animal model provided a reliable means of assessing heterotopic ossification severity and type.
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Influence of Limb Selection and Blast Medium on Heterotopic Ossification after Blast Amputation in a Rat Model

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1 Influence of Limb Selection and Blast Medium on Heterotopic Ossification after Blast

2 Amputation in a Rat Model

3

Abstract

Background: Heterotopic ossification develops after nearly two-thirds of traumatic blast amputations that occur in the contemporary battlefield. We used a Sprague-Dawley rat model, without the need for addition of an exogenous osteogenic agent, to evaluate the pathophysiology of ectopic bone formation in a traumatic blast amputation setting. We investigated the comparative frequency, quality, and quantity of heterotopic bone developing after blast amputation under various conditions.

Methods: Forty-eight animals underwent blast amputation and primary surgical closure (twenty-four forelimbs and twenty-four hind limbs), with only marginal skin débridement. Half of the amputations in each designated limb group were subjected to blasted sand, and the other half were blasted with water. All animals were followed with serial radiographs until euthanasia at twenty-four weeks. Heterotopic bone severity and type were assessed at eight, sixteen, and twenty-four weeks by three independent graders using a novel grading scale.

Results: All animals developed radiographic evidence of heterotopic ossification without addition of exogenous osteogenic material. Overall, hind limbs developed more severe and more extensive ectopic ossification than did forelimbs, as evidenced by a greater propensity to develop ectopic bony islands in the soft tissues. No differences were observed in ectopic bone development when comparing blast amputation with sand versus blast amputation with water.

Conclusions: Hind limb amputation sites were more likely to develop severe heterotopic ossification and ectopic bony islands within the adjacent soft tissues than were forelimb amputations. Despite a trend toward increased risk of infection, sand-blasted animals did not develop substantially more ectopic bone than water-blasted animals. The grading scale

1 developed in this animal model provided a reliable means of assessing heterotopic ossification
2 severity and type.

3
4 **Clinical Relevance:** Heterotopic ossification has been simulated in an animal model without
5 addition of an osteogenic agent after blast amputation. The greater occurrence of ectopic bone in
6 the hind limb compared with the forelimb, regardless of the blasted medium, mimics clinical
7 observations in injured veterans and provides opportunity for further study. Differential response
8 of upper and lower limb to traumatic events may parallel known differences in extremity defense
9 mechanisms related to inflammatory reactions and propensity for infection.

1 **Introduction**

2 Recent conflicts in Iraq and Afghanistan have resulted in a large increase in the number of
3 combat-related amputations in soldiers. Improved battlefield casualty evacuation capabilities and
4 advances in protective equipment and body armor have led to a lower mortality rate for those
5 suffering limb-compromising events. As a result, the prevalence of wounded soldiers surviving
6 major battlefield injury has substantially increased in recent history, with greater than 88% of
7 reported events in the Iraq conflict being survivable¹. Unfortunately, the use of improvised
8 explosive devices has also increased during modern armed conflicts and continues to be the
9 weapon of choice during acts of terrorism. In the contemporary Middle East theatre, these facts
10 collectively account for the reality that nearly 70% of all injuries are isolated to the extremities
11 and that 70% to 80% of injuries are caused by explosive devices, resulting in high rates of
12 survivable combat-related amputations²⁻⁴. Having survived the initial trauma and resulting limb
13 amputation, soldiers face many potential complications that threaten to compromise their return
14 to productive civilian life. One such complication is the development of heterotopic ossification
15 within the residual limb.

16 Heterotopic ossification is the formation of mature, lamellar bone within non-osseous
17 tissues. This abnormal growth results from a disturbance in the regulation of normal
18 skeletogenesis. This aberrant biological process is frequently encountered in other orthopaedic
19 settings and commonly observed after total hip arthroplasty and elbow fracture⁵⁻¹² and after
20 traumatic brain injury and spinal cord injury^{13,14}. Nevertheless, heterotopic ossification in cases
21 of combat-related amputations has received little attention throughout history. Despite being
22 documented as far back as the Civil War and the First World War, very few comprehensive
23 reports on its frequency in combat-related injuries exist. Recently, Potter et al.^{15,16} examined

1 rates of ectopic bone formation in cases of battlefield amputations and found a prevalence
2 approaching nearly two-thirds of veteran amputees. Compared with non-blast mechanisms of
3 injury, blast amputations correlated with increased rates of heterotopic ossification (66% versus
4 42%)¹⁵. Another previous study¹⁷ found similarly high rates of ectopic bone in blast amputees.
5 Such high rates of heterotopic ossification are clinically important because ectopic bone growth
6 is painful and devastating for the wounded soldier. Complications related to heterotopic bone in
7 residual limbs include pain, overlying skin and muscle breakdown, poor prosthetic fitting and
8 function, a need for surgical stump revision, and delayed rehabilitation¹⁶. Current treatment
9 options are limited and are restricted to surgical excision of the offending bone rather than
10 prevention¹⁸. Investigation of the effects of blast trauma on the musculoskeletal system,
11 specifically the causative mechanism and prevention of heterotopic ossification in the residual
12 limbs of blast amputees, is a priority in the care of war-wounded veterans.

13 Our objective was to evaluate factors that influence the development of ectopic bone
14 within the residual limb of a blast-injured rat. Specifically, the comparative frequency, quantity,
15 and quality of ectopic bone growth after blast amputation of hind limbs and forelimbs were
16 compared under two different blast conditions: sand and water. Considering the increased muscle
17 bulk in hind limbs and the potential for greater contamination, we hypothesized that more
18 ectopic bone would develop in hind limbs subjected to blast amputation with sand.

20 **Materials and Methods**

21 The study was conducted under a protocol approved by our Institutional Animal Care and Use
22 Committee and was ***Blinded by JBJS***. Forty-eight male Sprague-Dawley rats that were
23 twelve to fourteen weeks old underwent blast amputation and immediate surgical closure, with

minimal débridement limited to charred skin edges to facilitate primary wound healing. Twenty-four of the animals underwent left hind limb amputation and twenty-four underwent left forelimb amputation according to a previously refined protocol. Twelve animals in each extremity group underwent amputation via a column of blasted sand; the other twelve underwent blast amputation with propelled water. After wound closure, postoperative anteroposterior and lateral view radiographs of the residual limb were obtained as baseline images. The rats were maintained for twenty-four weeks, and serial orthogonal radiographs of the residual limb were obtained at ten days and at four, eight, twelve, sixteen, twenty, and twenty-four weeks. Euthanasia was planned for all animals at twenty-four weeks.

Blast Amputation Procedure and Monitoring

Inhaled isoflurane anesthesia was induced and maintained. Buprenorphine (0.05 mg/kg) and enrofloxacin (5 mg/kg) were administered subcutaneously for preemptive analgesia and prophylactic antibacterial coverage, respectively. Preemptive administration of 6 to 10 mL of warmed normal saline was provided subcutaneously for volume support. The selected limb (either left hind limb or left forelimb) was cleared of hair with a clipper and cleansed with chlorhexidine and 70% isopropyl alcohol. Maintaining deep inhalation anesthesia by nose cone, the rat was positioned prone on a 2-inch-thick aluminum platform that features a 2.5-inch hole in its center; the animal was tightly secured with industrial strength Velcro (Fig. 1). The selected limb was positioned across the hole and centered at the desired amputation level with the use of a silk suture and duct tape. The platform was located above a 2 foot × 2 foot × 2 foot steel tank filled with tap water or commercially purchased wet sand. An explosive charge (0.75 g of pentaerythritol tetranitrate) was submerged below the surface of the water or sand at a calibrated

1 distance to provide an equivalent force at the injury site, directly beneath the center of the hole in
2 the platform. The explosive was detonated with the use of a commercially available detonation
3 box. The resulting chemical reaction created a very large volume of hot, high-pressure gases that
4 acted against the surrounding medium, accelerating the blasted medium upward at velocities
5 approaching four times the speed of sound in air (Mach 4). These very large velocities were
6 converted into pressure upon impact with both the plate and the animal's exposed extremity.
7 Pressures on the order of tens of thousands of pounds per square inch were absorbed by the limb,
8 which was quickly and cleanly amputated. The protective platform effectively shielded the
9 animals from injury to internal organs, and survival of the blast was nearly universal.

10 After amputation, the animals were maintained on inhaled isoflurane anesthesia and
11 rapidly transferred to a nearby sterile operating table. Modest char was observed on the soft
12 tissues, and minimal blood loss occurred because of cauterization of the vessels from the high
13 temperature and pressure of the blast. The wound was irrigated with 150 to 250 mL of a 40:1
14 saline:chlorhexidine solution with a bulb syringe. Only prominent bone spikes were débrided.
15 Damaged muscle was inverted without débridement, and a primary fascial closure was
16 accomplished by oversewing with a running 4-0 Vicryl suture. The charred skin edges were
17 sharply and minimally débrided to facilitate primary wound healing and were stapled and sealed
18 with Histoacryl tissue adhesive (B. Braun Corporation, Bethlehem, PA). The animal was placed
19 in a clean cage with warmed bedding and was monitored until awakening from anesthesia.

20 Animals received antibiotics (enrofloxacin, 5 mg/kg administered subcutaneously twice a
21 day) for a total of three days. Analgesia was provided (buprenorphine, 0.05 mg/kg administered
22 subcutaneously on a standing "around the clock" regimen) for five days after the procedure.
23 Additional analgesia was provided as indicated by physiological signs of animal discomfort.

1

2 *Data Collection and Statistical Analysis*

3 After the animals were euthanized at twenty-four weeks, three independent graders reviewed the
4 series of radiographs and assessed the severity of heterotopic bone as absent, mild, moderate, or
5 severe based on a modification of the scale used by Potter et al.¹² for human amputees. In hind
6 limbs, heterotopic ossification was considered to be mild if it measured less than 25% of the
7 width of the tibial plateau on either the anteroposterior or lateral view radiograph, moderate if it
8 measured 25% to 50%, and severe if it measured more than 50%. For forelimbs, the same
9 grading system was applied with the distal humerus as the comparator.

10 The ectopic bone was classified as one of three types. Type 1 was defined as bone
11 growing contiguously with the residual limb while remaining within the normal bony
12 configuration. Type 2 was defined as ectopic bone contiguous with the residual limb skeleton but
13 outside the normal bony envelope. Type 3 was defined as ectopic bone originating within the
14 surrounding soft tissues of the residual limb but not contiguous with the residual bony anatomy.

15 The three graders independently evaluated the radiographs of all forty-eight animals and
16 assessed the severity and type of ectopic bone at three distinct time points: eight, sixteen, and
17 twenty-four weeks after injury. The graders were blinded to which medium was involved in the
18 blast.

19 Analysis of the data included the calculation of means and 95% confidence intervals
20 (95% CI) for heterotopic ossification severity by anatomic location and Wilcoxon rank-sum tests
21 to determine significant differences in severity. Severity grades were then dichotomized into
22 moderate and/or severe and mild and/or none categories to determine whether the frequency of
23 clinically meaningful heterotopic ossification differed by extremity location. Fisher's exact test

was used to determine significant differences in dichotomized heterotopic ossification severity. Cronbach's alpha statistic was used to determine internal consistency among the three independent raters of severity and type based on radiographs. Significance was assessed at an alpha level of 0.05, and all p-value calculations were two-sided.

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*****Blinded by JBJS*****

Results

One rat did not survive forelimb blast amputation because of an anesthetic overdose; it was replaced after Institutional Animal Care and Use Committee approval of a protocol amendment, leaving a total of forty-eight animals that survived the blast and surgical wound closure procedure. One hind limb amputation in the water group underwent revision for tibial overgrowth of ectopic bone with puncture through the skin four weeks after injury. The revision included minimal shortening of the remaining tibia with a rongeur, copious irrigation with sterile saline by syringe, and reclosure of the fascial layer; no signs of gross infection were evident. The animal ultimately developed severe heterotopic ossification (type 3, unanimously graded by all three graders), by the time of euthanasia at twenty-four weeks.

Three rats that had undergone sand blasting of a hind limb developed postoperative wound infections. These animals underwent irrigation and débridement of the wound followed by immediate reclosure. Two of these animals developed persistent infection and were euthanized at nine weeks; both developed severe, type 3 heterotopic ossification by the time of euthanasia (unanimously graded by all three graders). The other infection resolved and the

1 animal was euthanized at twenty-four weeks with only mild, type 1 heterotopic ossification
2 (unanimously graded by all three graders).

3 All other animals survived for the planned period of 24 weeks without requiring
4 reoperation or incurring other complications. No statistically significant difference was observed
5 in infection rate between blast amputations made with water and those made with sand (zero of
6 twenty-four versus three of twenty-four, $p = 0.23$) or between hind limbs and forelimbs (three of
7 twenty-four versus zero of twenty-four, $p = 0.23$).

8 Each *severity* grade of heterotopic ossification was assigned a corresponding numerical
9 value (absent = 0, mild = 1, moderate = 2, severe = 3). If disagreement existed among the
10 reviewers, the score assigned with greatest frequency (i.e., by two of three raters) was used.
11 Cronbach's alpha test was conducted to assess internal consistency among the graders of
12 heterotopic ossification. For radiographic heterotopic bone *severity*, an overall alpha statistic of
13 0.93 indicated excellent internal consistency among the three graders at all time points evaluated
14 regardless of the extremity amputated. Regarding the *quality* of heterotopic bone (described as
15 Type 1, 2, or 3), the Cronbach's alpha statistic was 0.78.

16 At twenty-four weeks, the mean heterotopic ossification *severity* of all forelimb
17 amputations was 1.25 (95% CI: 1.07 to 1.43) compared with a mean severity of 2.08 (95% CI:
18 1.72 to 2.44) for all hind limbs ($p < 0.001$). Likewise, moderate to severe heterotopic ossification
19 was found in sixteen (67%) of twenty-four amputated hind limbs (Figs. 2 and 4) compared with
20 six (25%) of twenty-four amputated forelimbs (Figs. 3 and 5) ($p = 0.008$). Corresponding three-
21 dimensional computed tomographic scans further outlined the bony anatomy. Among the twenty-
22 four hind limbs, ten (42%) developed severe ectopic bone, compared with zero forelimbs ($p <$
23 0.001). Extremities blasted with sand developed an average heterotopic ossification *severity* of

1 1.58 (95% CI: 1.26 to 1.90), and water-blasted extremities developed a mean heterotopic
2 ossification *severity* score of 1.75 (95% CI: 1.40 to 2.10; $p = 0.49$). In general, the amount of
3 heterotopic bone evident on radiographs continued to progress over time in each experimental
4 group (Fig. 6).

5 An animal was deemed to have type 3 heterotopic ossification if two of three graders
6 identified a bony island. Twenty-one hind limbs developed type 3 ectopic bone compared with
7 eleven forelimbs ($p = 0.005$). Sixteen animals in the sand-blasted group and six animals in the
8 water-blasted group developed Type 3 heterotopic ossification ($p = 1.00$).

10 Discussion

11 Between 2001 and 2005, more than 3500 extremity wounds were recorded in the Joint Theater
12 Trauma Registry. These injuries were evenly distributed between upper and lower extremities,
13 and 75% were the result of explosive munitions^{4,19}, yet our institutional experience has suggested
14 that the upper limb may be relatively protected from heterotopic ossification. To further explore
15 the phenomenon of ectopic bone formation occurring after blast amputation, an animal model
16 was developed to replicate this process without the addition of exogenous osteogenic agents. Our
17 previous work demonstrated high survivability of a controlled blast amputation of a single
18 extremity in a Sprague-Dawley rat model²⁰.

19 In this study, all animals developed some degree of heterotopic ossification after blast
20 amputation. Compared with residual forelimbs, residual hind limbs developed more severe
21 heterotopic bone and were more likely to exhibit ectopic bony islands in the surrounding soft
22 tissues. On average, the hind limb developed one full grade more severe heterotopic ossification
23 than did a typical forelimb blast amputation. These observed differences correlate closely with

clinical observations in humans²¹. Forsberg et al.¹⁷ reported incidences of ectopic bone formation in lower and upper extremity wartime amputations of 66% and 30%, respectively. Similarly, while studying a different mechanism of amputation, Helm and Walker²² reported that 78% of upper extremity electrical burn amputations developed substantial ectopic bone compared with 90% of lower extremity amputations.

Our modified human radiographic grading scale for heterotopic ossification *severity* demonstrated excellent overall internal consistency (Cronbach's alpha = 0.93), validating it as a reliable means with which to grade heterotopic ossification severity in the rat experimental model. Overall, plain radiographic determination of heterotopic ossification *type* was also reliable (Cronbach's alpha = 0.78), but reliability for type was slightly weaker than for severity. We identified some potential reasons for disagreement among reviewers. Regarding heterotopic ossification type, disagreement likely developed in determination of whether bone observed in the soft tissue was a retained bony fragment or truly new ectopic bone. After forelimb blast amputation, many small fragments of bone often remained within the extremity. The small bones of the forelimb typically shattered into many pieces, making it difficult to ascertain whether bone in the surrounding soft tissue represented fragments retained from the initial injury or newly formed islands of ectopic bone. Additionally, because of the close proximity of the forelimb to the animal's chest, radiograph resolution was compromised by motion artifact caused by normal respiratory effort. Also, true anteroposterior view films of the distal forelimb were difficult to obtain because of positioning of the flexed elbow during film acquisition. For these reasons, determinations in the hind limb were more reliable and had greater inter-observer consistency. These limitations were mitigated by performing immediate post-blast and serial computed tomography, which allowed for accurate comparative computation of newly developed bone.

1 Notwithstanding these limitations of radiography, our radiographic classification scheme was
2 reliably consistent among graders and is suitable for use with future experiments.

3 Differences in hind limb versus forelimb heterotopic ossification after blast amputation
4 may provide insight into the pathophysiology of ectopic bone formation as it relates to the
5 possible mechanistic role of an underlying inflammatory process. In humans, conventional
6 wisdom holds that lower extremity wounds are more susceptible to infection than are upper
7 extremity wounds. After controlled inoculation of bacteria in different sites in human volunteers,
8 Duncan et al.²³ demonstrated infection rates of 13% and 38% in the arm and thigh, respectively.
9 Lineaweaver et al.²⁴ later demonstrated lower extremities to be relatively deficient in neutrophil
10 recruitment and delivery to local wound sites compared with upper extremities after controlled
11 local bacterial infection in normal volunteers. Both qualitative and quantitative differences in the
12 physiological inflammatory response in upper and lower limbs may likewise play a role in the
13 differential frequency of ectopic bone observed in humans and in this animal model.

14 Furthermore, in the rat, myocutaneous soft-tissue cover of the bones of the distal hind
15 limb is more robust than in the distal forelimb (humans similarly have more muscle mass in the
16 lower extremities). Accordingly, more muscle injury might reasonably be expected after hind
17 limb compared with forelimb blast amputation; this may influence the amount of tissue necrosis,
18 resultant inflammation, and subsequent ectopic bone formation. It is thought that muscle injury
19 and inflammation are critical initiators of heterotopic ossification because they induce expression
20 of bone morphogenic protein. Mesenchymal stem cells respond to local bone morphogenic
21 proteins and are induced to differentiate into osteoblasts, leading to new bone formation. This
22 paracrine action of bone morphogenic proteins may account for observed differences in ectopic
23 bone formation after local injury²⁵⁻²⁷. Although rats are quadrupeds, they preferentially put

weight on their hind limbs when rearing. With increased weight bearing on their residual hind limbs, the animals may have stimulated more local inflammation and tissue damage, which could have further potentiated the process of heterotopic ossification in the hind limb.

Interestingly, no difference in heterotopic ossification quality or type was observed among extremities amputated with sand compared with those amputated with water. We expected to see higher rates of infection and wound complications in the sand group, with an associated increase in ectopic bone development related to a general amplification of the local inflammatory response. Although three wound infections occurred in the sand hind limb group, this infection rate was not statistically greater than in the water blast group. The blasting standoff distances in each group were calibrated such that both media imparted a comparable amount of energy to the extremity, resulting in similarly clean amputations. The findings from this experiment imply that the physical trauma caused by the blast mechanism itself leads to ectopic bone formation, regardless of the medium used or any potentiation of the inflammatory response resulting from concurrent infection or delayed wound healing.

This work demonstrates a clear predilection for heterotopic ossification after hind limb blast amputation in a Sprague-Dawley rat model, which might be attributable to an amplified or preconditioned inflammatory response, greater muscle tissue injury, or both in the hind limb. We anticipate that future investigations will elucidate similarities between soldiers' wartime extremity injuries and this animal model that have beneficial therapeutic implications.

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1 **Figure Legends**

2 Fig. 1

3 Setup for a blast amputation of the left hind limb of a Sprague-Dawley rat.

4

5 Fig. 2

6 Immediate post-blast anteroposterior view (a) and lateral view (b) radiographs of a residual hind
7 limb following blast amputation.

8

9 Fig. 3

10 Immediate post-blast anteroposterior view (a) and lateral view (b) radiographs of a residual
11 forelimb following blast amputation.

12

13 Fig. 4

14 Plain radiographs (a and c) and computed tomographic three-dimensional reconstructed images
15 (b and d) obtained twenty-four weeks after blast show a residual hind limb following blast
16 amputation with severe type 3 heterotopic bone.

17

18 Fig. 5

19 Plain radiographs (a and c) and computed tomographic three-dimensional reconstructed images
20 (b and d) obtained twenty-four weeks after blast show a residual forelimb following blast

1 amputation graded as having mild heterotopic ossification. The ossification was classified as
2 type 1 based on radiographs and type 2 based on more detailed computed tomographic images.

3

4 Fig. 6

5 Average heterotopic ossification severity of each group of rats is shown at three distinct time
6 points. A progressive increase in the observed severity of heterotopic ossification occurred over
7 time until euthanasia at twenty-four weeks. A severity score of 1 corresponds to mild heterotopic
8 ossification, whereas scores of 2 and 3 represent moderate and severe heterotopic ossification,
9 respectively. Error bars represent 95% confidence intervals.

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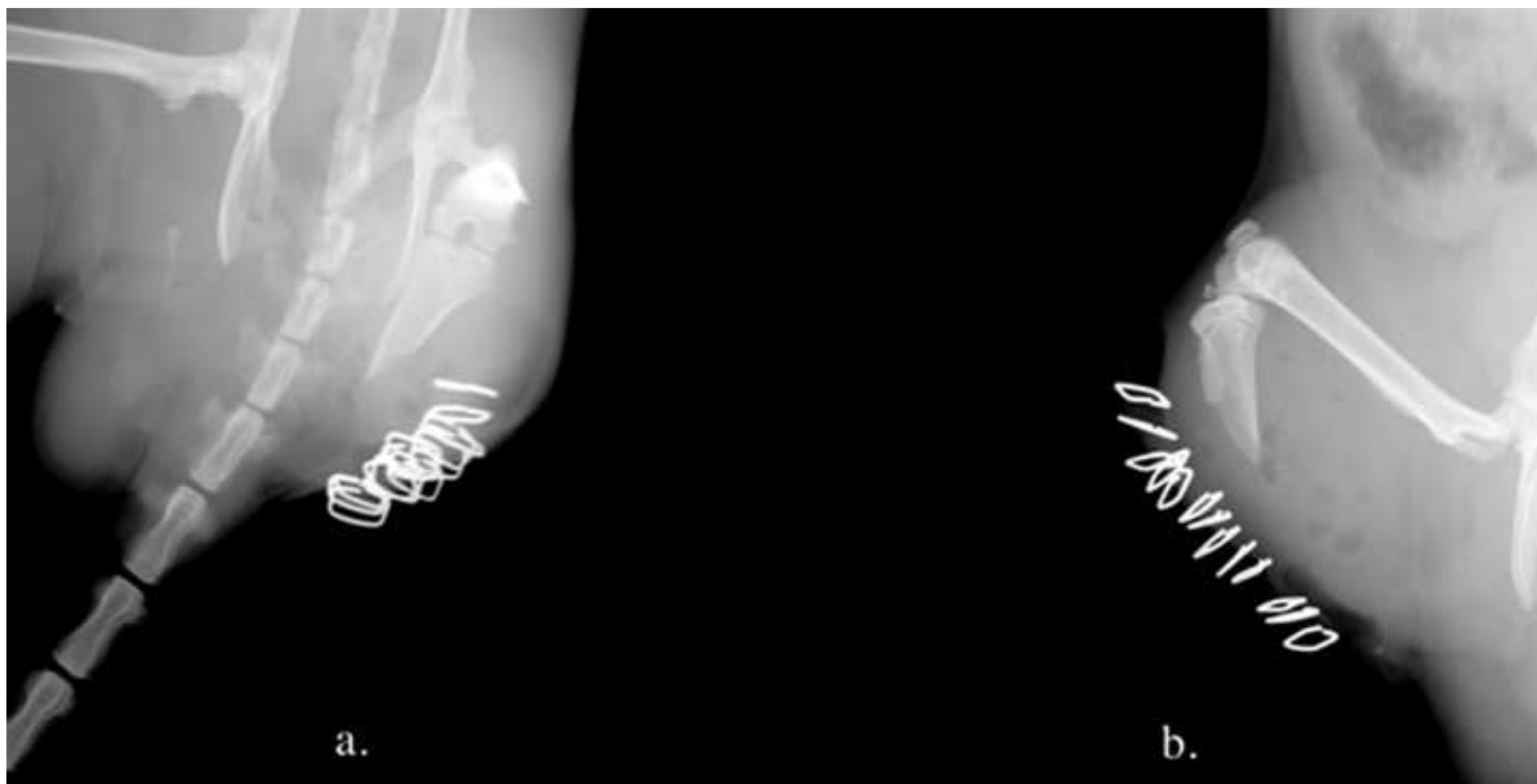


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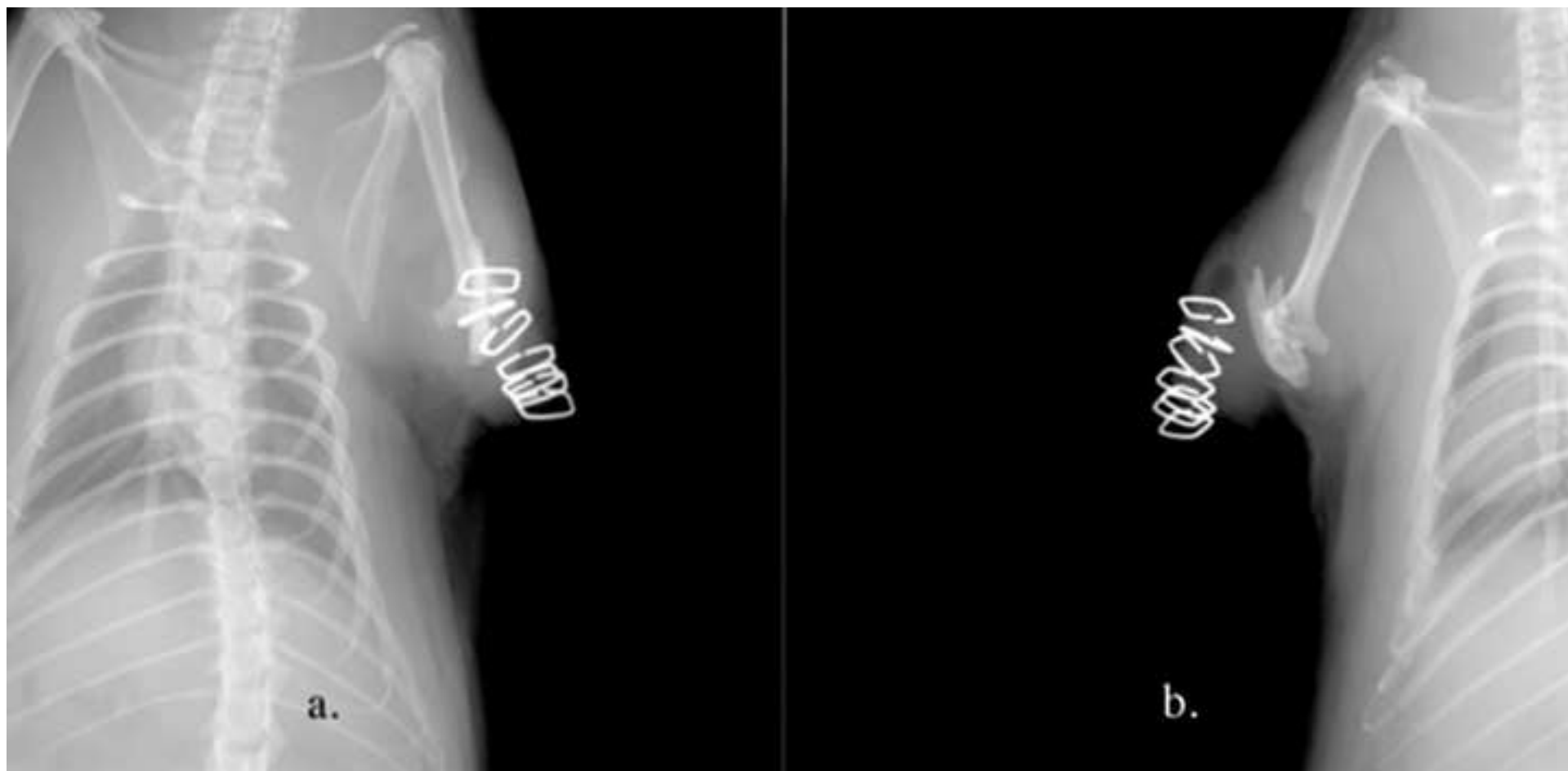


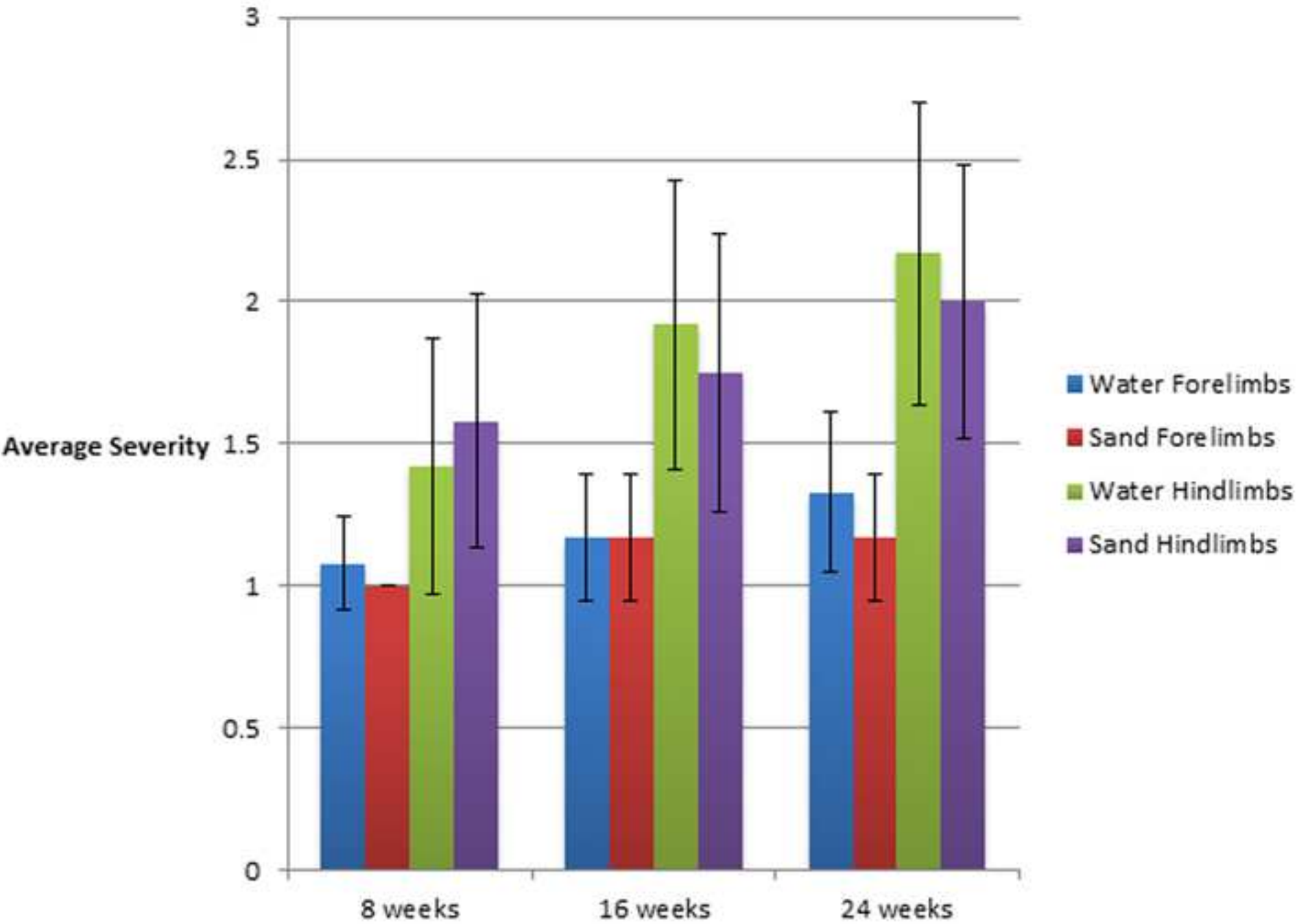
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Date _____ 7/21/2012

Signature _____

Name (please print) _____

Date _____

Signature _____

Name (please print) _____

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Signature David Jin Yoo

Name (please print) David Jin Yoo

Date 07/30/2012

Signature _____

Name (please print) _____

Date _____

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Name (please print) Jason Blevins

Date 7/24/12

Signature _____

Name (please print) _____

Date _____

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Name (please print) _____

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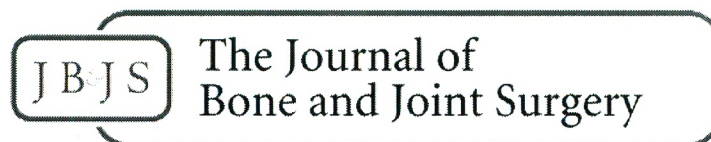
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Name (please print) Gregory V. Gasbarro

Date 7/19/12

Signature _____

Name (please print) _____

Date _____

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Signature Tyler Hughes

Name (please print) Tyler Hughes

Date 7/25/2012

Signature _____

Name (please print) _____

Date _____

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Name (please print) _____

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Name (please print) _____ Ebrahim Paryavi

Date _____ 7-21-2012

Signature _____

Name (please print) _____

Date _____

Signature _____

Name (please print) _____

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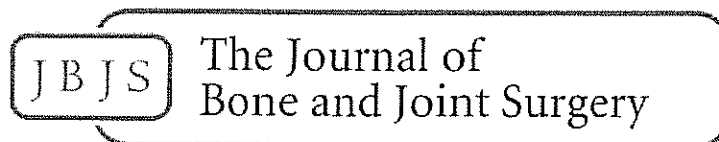
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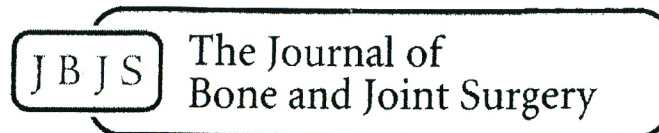
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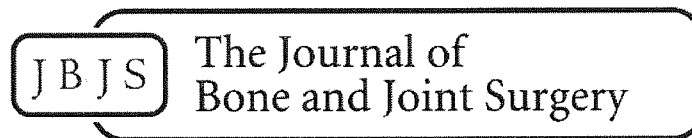
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